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Short communication

Ethanol-induced c-Fos expression in rat lines selected for low and high alcohol consumption

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Abstract

Selectively bred rat lines, developed to model genetic contributions to alcohol abuse, include the Indiana alcohol-preferring (P) and alcohol-nonpreferring (NP) lines, and the Alko-Alcohol (AA) and Alko-Nonalcohol (ANA) lines. Preferring and nonpreferring lines were compared in their response to intraperitoneal injection of either ethanol or isotonic saline using c-Fos expression as a marker of neuronal activity. Although line differences were noted in several brain regions, the principal finding was that alcohol-nonpreferring lines (NP and ANA) displayed greater c-Fos expression in the locus coeruleus (LC) relative to the alcohol-preferring lines (P and AA) following injection of 3.0 g ethanol/kg. These data point to the LC as an area which may play a role in the differences in voluntary ethanol consumption between rat lines genetically bred for low and high ethanol preference.

Keywords: Ethanol; c-Fos; Genetics; Selected line; Locus coeruleus; Nucleus accumbens

One of the first rat models developed to study genetic contributions to alcoholism includes the Indiana alcohol-preferring (P) and alcohol-nonpreferring (NP) rat lines, which were selectively bred for differences in consumption of a 10% (v/v) ethanol solution [11]. When given a choice between water and 10% alcohol, P rats will freely drink enough alcohol to produce intoxication [13], while NP rats almost totally avoid a 10% ethanol solution [10]. Similar differences have been observed between the alcohol-preferring AA (Alko, Alcohol) and the alcohol-nonpreferring ANA (Alko, Nonalcohol) rat lines [4].

Although baseline differences in neural systems are suggestive (e.g. [12]), their role in the differences in alcohol intake and preference between selected lines remains unclear. An alternative approach is to examine patterns of neuronal activation in response to ethanol exposure, to identify potential line differences in response to the drug. Neuronal activity can be assessed by immunostaining for c-Fos, the protein product of the immediate-early gene c-*fos* [16], which increases in accordance with neuronal activation. Recently, c-Fos immunohistochemistry has been used to identify neuronal sites activated by ethanol exposure in rats [2,15,18,20]. In the present research, c-Fos-like immunoreactivity (cFLI) was used to assess differences in neuronal activity between the P and NP lines following intraperitoneal (IP) injection of either ethanol or isotonic saline. It was hypothesized that differences might be evident in those regions thought to be associated with the reinforcing effects of ethanol, such as the nucleus accumbens (Acb) [9], and brainstem regions thought to mediate the aversive effects of ethanol, including the area postrema (AP), the nucleus of the solitary tract (NTS), the lateral parabrachial nucleus (PBN), and the locus coeruleus (LC) [14,18]. To assess the generality of our findings with P and NP rats, the AA and ANA lines were also studied.

Alcohol-naïve adult male P and NP rats (F_{39-40} generations), weighing between 350 and 500 g on the test day, were obtained from the Indiana University School of Medicine. Alcohol-naïve adult male AA and ANA rats (F_{72} generation) weighed between 300 and 400 g on the test day and were obtained from the Biomedical Research Center of Alko Ltd. (Helsinki, Finland). Rats were allowed to habituate to the laboratory and to handling for at least 3 weeks before the experiment was initiated.

Rats from preferring and nonpreferring lines were distributed to groups equated for body weight, and were given an i.p. injection of either 1.0 g ethanol/kg or 3.0 g ethanol/kg (25% (w/v), mixed in isotonic saline), or

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isotonic saline (12 ml/kg; this dose produced volumes equal to the largest dose of ethanol). This yielded a 2×3 (line $\times$ drug) experimental design ($n = 4$ rats/group) for each model studied. Rats were injected and returned to their home cage. Two hours following injection, rats were anesthetized with sodium pentobarbital (1.2 ml/kg, i.p.) and 2.0 ml blood samples were collected (via cardiac puncture) and processed for later analysis of plasma-ethanol levels (Sigma Diagnostics, Enzymatic Determination of Alcohol Test, St. Louis, MO). Rats were then transcardially perfused with isotonic phosphate-buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, and brains were removed and postfixed for approximately 24 h.

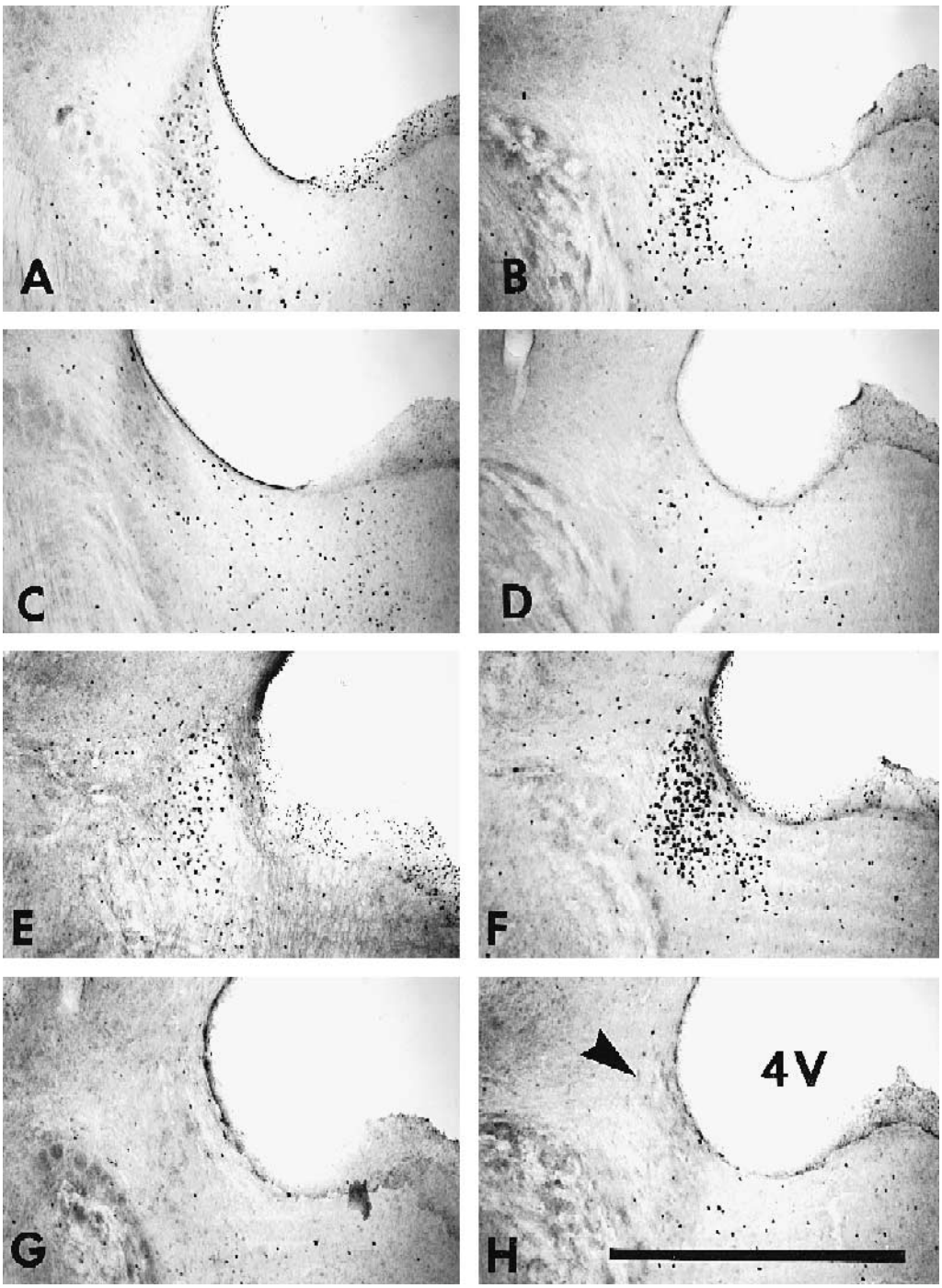

Fig. 1. Representative photomicrographs showing slices through the LC (arrow). A,B: alcohol-preferring P rats (A) and alcohol-nonpreferring NP rats (B) given injection of 3.0 g ethanol/kg. C,D; P rats (C) and NP rats (D) given injection of isotonic saline. E,F: alcohol-preferring AA rats (E) and alcohol-nonpreferring ANA rats (F) given injection of 3.0 g ethanol/kg. G,H: AA rats (G) and ANA rats (H) given injection of isotonic saline. Also shown is the fourth ventricle (4V). The LC is approximately -10.6 mm relative to bregma. Scale bar is approximately 0.38 mm.

Seventy-five μm slices were cut from the brain with a vibrotome. Forebrain slices were made in the coronal plane to allow visualization of the Acb, as well as the central nucleus of the amygdala (CEA) and the paraventricular nucleus of the hypothalamus (PVN), structures that express cFLI following ethanol exposure. Brainstem slices were made in the horizontal plane to allow visualization of the rostro-caudal extent of the NTS, the AP, the PBN, and the LC. Tissues were prepared for cFLI by immunohistochemistry according to methods described elsewhere [18]. Camera lucida drawings were made in an area of approximately 0.38 mm^2 by an experimenter unaware of group treatments. Care was taken so that structures were scored in approximately the same plane. Drawings were scored by a blind rater who recorded the number and location of c-Fos-positive nuclei. Because this study was designed, a priori, to examine differences between preferring and nonpreferring lines, Student's *t*-tests were used to analyze line differences at each level of the Drug factor [19]. In all cases, $P < 0.05$ (two-tailed) indicated statistical significance.

cFLI from P and NP lines: photomicrographs of the LC from P and NP rats are shown in the top half of Fig. 1A–D, while average nuclei positive for cFLI in all examined brain regions of P and NP lines are presented in Table 1. As suggested by these data, NP rats showed significantly greater cFLI in the LC relative to P rats following injection of 3.0 g ethanol/kg [$t(6) = 3.25$]. Following injection of 1.0 g ethanol/kg, cFLI in the core region of the Acb was significantly greater in P rats than in NP rats [$t(6) = 3.28$].The NP line demonstrated levels of cFLI in the NTS that were significantly greater than in the P line following injection of either saline [$t(6) = 3.05$] or 3.0 g ethanol/kg [$t(6) = 4.00$]. In all other brain regions that showed cFLI, no significant differences were found between lines.

Table 1
Average number of nuclei positive for cFLI in P and NP lines following drug injection

	Saline	Ethanol dose	
		1.0 g/kg	3.0 g/kg
Acb-core			
(P)	106.25 ± 27.68	130.75 ± 15.81 [a]	122.00 ± 30.25
(NP)	88.25 ± 23.05	65.75 ± 11.93	92.00 ± 14.06
Acb-shell			
(P)	95.00 ± 19.74	61.75 ± 11.03	58.75 ± 09.62
(NP)	64.00 ± 09.44	53.50 ± 05.17	58.50 ± 05.56
CEA			
(P)	38.25 ± 10.27	276.25 ± 28.82	311.00 ± 25.71
(NP)	24.75 ± 05.28	210.50 ± 27.08	311.00 ± 22.72
PVN			
(P)	149.25 ± 40.25	253.25 ± 80.26	651.00 ± 84.50
(NP)	258.50 ± 74.89	313.75 ± 54.04	662.50 ± 107.81
LC			
(P)	36.25 ± 15.58	52.25 ± 31.86	41.75 ± 19.73 [a]
(NP)	63.75 ± 25.77	37.00 ± 07.93	119.75 ± 13.61
PBN			
(P)	56.00 ± 11.05	127.00 ± 28.01	308.75 ± 29.65
(NP)	120.25 ± 27.36	211.75 ± 31.20	330.00 ± 34.94
NTS			
(P)	37.75 ± 07.89 [a]	215.25 ± 71.81	211.50 ± 29.85 [b]
(NP)	122.50 ± 26.60	281.75 ± 76.99	381.25 ± 30.13
AP			
(P)	127.25 ± 19.78	352.50 ± 55.21	549.50 ± 58.09
(NP)	178.75 ± 45.41	463.00 ± 60.89	597.25 ± 181.08

Data are the means ± S.E.M. for P ($n = 4$/drug) and NP ($n = 4$/drug) lines.
[a] Significant at $P < 0.05$ relative to NP.
[b] Significant at $P < 0.01$ relative to NP.

Table 2
Average number of nuclei positive for cFLI in AA and ANA lines following drug injection

	Saline	Ethanol dose	
		1.0 g/kg	3.0 g/kg
Acb-core			
(AA)	61.75 ± 21.25	34.25 ± 11.86	56.50 ± 11.65
(ANA)	33.50 ± 09.68	65.50 ± 16.78	76.50 ± 15.21
Acb-shell			
(AA)	51.50 ± 06.66	47.00 ± 10.58	40.00 ± 07.87 [a]
(ANA)	34.25 ± 02.98	46.00 ± 08.95	73.00 ± 09.60
CEA			
(AA)	64.50 ± 10.85	244.00 ± 22.21	290.25 ± 19.15
(ANA)	63.00 ± 18.66	208.25 ± 54.00	248.00 ± 31.89
PVN			
(AA)	78.00 ± 14.58	195.75 ± 74.48	621.25 ± 72.41
(ANA)	63.75 ± 12.20	147.50 ± 47.04	582.50 ± 73.89
LC			
(AA)	14.75 ± 03.75	21.25 ± 09.62	73.00 ± 09.28 [a]
(ANA)	10.75 ± 00.75	22.00 ± 04.53	134.25 ± 24.69
PBN			
(AA)	45.50 ± 04.05	143.00 ± 33.53	212.25 ± 22.31
(ANA)	50.50 ± 09.33	190.00 ± 20.01	221.75 ± 12.66
NTS			
(AA)	54.75 ± 09.95	107.50 ± 41.88	216.75 ± 22.55
(ANA)	37.75 ± 03.04	141.50 ± 11.93	264.25 ± 43.73
AP			
(AA)	100.75 ± 13.73	336.50 ± 56.55	443.25 ± 23.07
(ANA)	129.75 ± 21.68	229.00 ± 39.83	467.00 ± 15.03

Data are the means ± S.E.M. for AA ($n = 4$/drug) and ANA ($n = 4$/drug) lines.
[a] Significant at $P < 0.05$ relative to ANA.

cFLI from AA and ANA lines: photomicrographs of the LC from AA and ANA rats are shown in the bottom half of Fig. 1E–H, and average numbers of nuclei positive for cFLI in brain regions of AA and ANA lines are presented in Table 2. In the LC, the nonpreferring ANA line displayed significantly greater cFLI than the preferring AA line after injection of 3.0 g ethanol/kg kg [$t(6) = 2.40$]. In the shell region of the Acb, ANA rats showed significantly greater cFLI than AA rats after injection of 3.0 g ethanol/kg [$t(6) = 2.66$]. No other line differences were observed.

Plasma-ethanol data: analysis of plasma-ethanol levels revealed that there were no differences between P and NP lines 2 h following injection of 1.0 g ethanol/kg (6.31 ± 4.48 mg/dl and 16.19 ± 5.18 mg/dl, respectively), nor were there differences 2 h following injection of 3.0 g

ethanol/kg (190.56 ± 9.97 mg/dl and 179.75 ± 7.06 mg/dl, respectively). Likewise, no significant differences existed between AA and ANA lines 2 h following injection of 1.0 g ethanol/kg (11.94 ± 10.54 mg/dl and 38.31 ± 15.00 mg/d, respectively) or 2 h following injection of 3.0 g ethanol/kg (183.63 ± 9.07 mg/dl and 188.69 ± 3.26 mg/dl, respectively).

The one consistent effect across both models was that injection of a relatively high dose of ethanol (3.0 g/kg) induced cFLI in the LC, and that cFLI in this region was significantly higher in both nonpreferring lines (NP and ANA) than in the preferring lines (P and AA). Because plasma-ethanol levels did not differ significantly between lines 2 h following injection, the present data cannot be accounted for by differences in circulating levels of ethanol or patterns of ethanol elimination. These data point to the LC as an area which may contribute to differences in voluntary ethanol consumption between rat lines genetically bred for low and high ethanol preference, an observation strengthened by the fact that differences emerged *following* exposure to ethanol, rather than being baseline differences in the absence of the drug.

The LC is the major noradrenergic nucleus in the brain [5], and it appears to be activated during aversive states. During drug withdrawal [8], and during various forms of aversive stress, there are increased levels of cFLI in the LC [1]. Furthermore, the largest dose of ethanol used in the present study produced strong c-Fos expression in the LC, and similar doses have been found to reliably support a conditioned taste aversion [18]. Thus, higher levels of cFLI seen in the LC with increasing doses of ethanol may be a response to the aversive effects of the drug, a response that is more pronounced in the nonpreferring lines. Presumably, increased sensitivity to the aversive effects of ethanol in nonpreferring lines could reduce preference (and thus consumption) of that drug.

Differential c-Fos expression in the LC between lines may be related to differential activation of the endogenous opiate systems. The LC is rich in opiate receptors, and evidence indicates that opiate drugs, such as morphine, inhibit LC activity [14]. Recently, it has been shown that ethanol exposure stimulates the endogenous opiate system in P rats, but does little to activate the opiate system in NP rats [6]. Furthermore, AA rats have been found to have higher concentrations of brain-opiate receptors relative to ANA rats [3]. Thus, ethanol may have a greater inhibitory influence over the LC in the preferring line of rats, and this inhibition may enhance the reinforcing properties, and/or attenuate the aversive properties, of ethanol.

The Acb is thought to play a major role in the reinforcing effects of ethanol [9]. P rats had more cFLI in the core region of the Acb following injection of 1.0 g ethanol/kg than did NP rats; conversely, the ANA line displayed greater cFLI in the shell of the Acb when compared with the AA line, following the 3.0 g/kg dose of ethanol. Since patterns of Acb activation were not consistent across the two models, results could be unrelated to differences in alcohol preference. Finally, because nonpreferring lines appear to be more sensitive to the aversive properties of ethanol [7], it was predicted that these lines might display greater levels of ethanol-induced cFLI in regions associated with aversion (the NTS, AP, and PBN [17,18]). Although the AA and ANA lines did not differ, the NP line did show greater cFLI in the NTS than the P line after injection of 3.0 g ethanol/kg, *but also* after injection of saline. Thus, differences between the P and NP lines in cFLI in the NTS may have been due to factors other than ethanol exposure (e.g. baseline differences in cFLI).

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